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The Activation Function-1 Domain of Nur77/NR4A1 Mediates Trans-activation, Cell Specificity, and Coactivator Recruitment*

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Nur77/NR4A1 is an "orphan member" of the nuclear hormone receptor superfamily. Nur77 and its close relatives Nurr1 and NOR-1 bind as monomers to a consensus binding site, the nerve growth factor induced protein I-B (NGFI-B)-binding response element (NBRE). The Nur77/NURR1/NOR1 nuclear receptors are classified as immediate early response genes which are induced through multiple signal transduction pathways. They have been implicated in cell proliferation, differentiation, and apoptosis. However, the mechanism of coactivation and ligand independent trans-activation remains unclear. Hence we examined the molecular basis of Nur77-mediated cofactor recruitment and activation. We observed that Nur77 trans-activates gene expression in a cell-specific manner, and operates in an activation function-1 (AF-1)-dependent manner. The AB region encodes an uncommonly potent N-terminal AF-1 domain delimited to between amino acids 50 and 160 and is essential for the ligand-independent activation of gene expression. Steroid receptor coactivator-2 (SRC-2) modulates the activity of the N-terminal AF-1 domain. Moreover, SRC-2 dramatically potentiates the retinoid induced RXR-dependent activation of the Nur77 ligand binding domain (LBD). Interestingly, the N-terminal AB region (not the LBD) facilitates coactivator recruitment and directly interacts with SRC, p300, PCAF, and DRIP-205. Consistent with this, homology modeling indicated that the Nur77 LBD coactivator binding cleft was substantially different from that of retinoic acid receptor γ, a closely related AF-2-dependent receptor. In particular, the hydrophobic cleft characteristic of nuclear receptors was replaced with a much more hydrophilic surface with a distinct topology. This observation accounts for the inability of this nuclear receptor LBD to directly mediate cofactor recruitment. Furthermore, the AF-1 domain physically associates with the Nur77 C-terminal LBD and synergizes with the retinoid X receptor LBD. Thus, the AF-1 domain plays a major role in Nur77-mediated transcriptional activation, cofactor recruitment, and intra- and intermolecular interactions.

Nuclear hormone receptors (NRs)[1] function as ligand-activated transcription factors that regulate gene expression involved in reproduction, development, and general metabolism (1). NRs function as the conduit between physiology and gene expression. The importance of NRs in human physiology is underscored by the extensive pharmacopoeia that has been created to combat disorders associated with dysfunctional hormone signaling. These diseases affect every discipline of medicine (2). All members of the NR superfamily display a highly conserved structural organization (1) with an N-terminal region AB (which encodes activation function-1, AF-1) followed by the C-region (which encodes the DNA binding domain (DBD)), a linker region D, and the C-terminal E region. The DE region encodes the ligand binding domain (LBD) and a transcriptional domain, denoted as activation function-2 (AF-2) (1, 2).

A decade ago, gene products were identified which appeared to belong to the NR superfamily on the basis of their nucleic acid sequence identity. The endogenous signaling molecules that bound to these proteins were unknown, and thus the term "orphan receptor" was coined. Based on members of the NR superfamily which have been characterized more fully, the orphans forecast an enormous yet unexploited opportunity for the discovery of important new therapeutic agents. The potential impact of such a discovery cannot be overstated because every known NR has been implicated in human disease.

The Nur77, Nurr1, and NOR-1 (NR4A1–3 subgroup) family of orphan NRs is well conserved in the DBD (~91–95%) and the C-terminal LBD (~60%), but it is divergent in the N-terminal AB region. This subgroup of proteins functions as immediate-early response genes that are induced by a wide range of physiological signals (3–7). They have been implicated in proliferation, differentiation, apoptosis, hypertrophy/remodeling, Parkinson's disease, schizophrenia, manic depression, and autoimmune disease (8–19). In itself, the NR4A1–3 subgroup presents an exciting scientific challenge, and unlocking the molecular mechanisms that mediate NR4A-dependent transcription will provide a potential platform for pharmacological intervention.

Nur77 has been shown to play a key role in regulating expression of various genes in the hypothalamic-pituitary-adrenal axis (HPA) related to inflammation and steroidogenesis (20–22). Specifically, Nur77 family members activate expression of corticotropin-releasing hormone (19). The Nur77 family can regulate steroid 21-hydroxylase, steroid 17-hydroxylase,

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[1] The abbreviations used are: NR(s), nuclear receptor(s); aa, amino acid(s); AF-1 and AF-2, activation function-1 and -2, respectively; CREB, cAMP-response element-binding protein; DBD, DNA binding domain; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione *S*-transferase; LBD, ligand binding domain; LUC, luciferase; NBRE, NGFI-B-binding response element; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SRC, steroid receptor coactivator; tk, thymidine kinase; GRIP-1, glucocorticoid receptor-interacting protein 1; pCAF, P300/CBP-associated factor; DRIP-205, vitamin D receptor-interacting protein complex component 205; TRAP, thyroid hormone receptor-associated protein; ARC, activator-recruited cofactor.

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and the 20α-hydroxysteroid dehydrogenase promoters (21–22). As such, the NR4A family of proteins represents a potential target for therapeutic intervention to control inflammatory responses.

Nur77 is expressed in skeletal muscle (23) and mouse myoblasts in culture (24, 26–27). Moreover, basic fibroblast growth factor, 12-*O*-tetradecanoylphorbol-13-acetate, and forskolin agonists induce Nur77 expression in mouse myoblasts. Early studies demonstrated that Nur77 expression in muscle appeared to be dependent to different extents on the activation of protein kinase A, C, and mitogen-activated protein kinase. β-Adrenergic agonists transiently induce Nur77 in mouse myoblasts (24, 26–27). Chronic stimulation of muscle with adrenergic agonists induces hypertrophy, fast type II fibers, and muscle remodeling, and it reduces skeletal muscle wasting (25).

The NR4A family members can bind as monomers and as homodimers to single/tandem copies of the NR4A response element, AAAGGTCA, a variant NR half-site, and constitutively regulate transcription (28–30). Moreover, Nur77 and Nurr1 (but not NOR-1) heterodimerize with RXR and mediate efficient trans-activation in response to RXR-specific agonists from DR5 motifs (31–33). The NR4A subgroup functions as immediate-early response genes, which suggests cross-talk between multiple classes of stimuli and retinoid-dependent signaling. Moreover, phosphorylation of Nur77 regulates retinoid signaling by controlling the subcellular localization and nucleocytoplasmic shuttling of RXR (7). In addition, serine and threonine residues in Nur77 have been demonstrated to be critical in the activity, function, and localization of the NR4A members (5, 7, 34, 35).

This receptor subgroup has proven to be refractory to the understanding of coactivator recruitment in the process of constitutive trans-activation and retinoid-induced RXR-dependent regulation (31–33). Moreover, the Nur77 LBD lacks an intrinsic and classical activation domain, and the serine/threonine-rich domain in the N terminus has been implicated in the regulation of Nur77-dependent transcription (36). Furthermore, the N-terminal AB region has been implicated in growth factor-dependent nucleo-cytoplasmic shuttling of this NR (7).

In summary, the N-terminal AF-1 region, the C-terminal LBD, and AF-2 domain of the Nur77 subgroup are very unusual. Nur77 lacks an archetypal AF-2 transcriptional activation domain and does not seem to interact with NR cofactors; notwithstanding these comments, this orphan modulates transcription. Currently, the molecular aspects of transcriptional activation remain obscure; furthermore, structural analysis of the LBD and mechanistic analysis of Nur77 function have not been undertaken with respect to intramolecular interactions and cofactor recruitment.

We have demonstrated and analyzed the functional role of AF-1 in mouse NR4A1 (Nur77)-mediated trans-activation, further elucidated the molecular basis of retinoid-induced RXR-dependent trans-activation by Nur77, and provided a transcriptional, biochemical, and structural analysis of coactivator function and recruitment.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—Proliferating C2C12 cells and COS-1 cells were kept in DMEM supplemented with 20 and 10% fetal calf serum (FCS), respectively, in 6% CO_2. Cells grown in 12-well dishes to 60–70% confluence were transiently transfected with 0.33–1 μg of pGL2b-tk-LUC (37) or pNBRE3-tk-LUC (31–33) reporter plasmid together with 0.33–0.5 μg of pSG5-Nur77FL or pSG5-Nur77ΔAB, or pSG5-Nur77ΔLBD or pSG5 alone using a DOTAP/DOSPER (Roche Molecular Biochemicals) liposome mixture in HEBS (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM Na_2HPO_4, 11 mM dextrose, pH 7.1) (total DNA 2–2.5 μg/well). The DNA/DOTAP/DOSPER mixture was added to the cells in 1 ml of fresh DMEM containing 10% FCS and incubated for 14 h. After transfection the medium was replaced, and the cells were grown a further 24–48 h. Cells were harvested and assayed for luciferase activity. Each experiment represented at least two sets of independent triplicates to overcome the variability inherent in transfection experiments (37–42).

Luciferase Assays—Luciferase activity was assayed using a Luclite kit (Packard) according to the manufacturer's instructions. Briefly, cells were washed once in phosphate-buffered saline and resuspended in 150 μl of phenol red-free DMEM and 150 μl of Luclite substrate buffer. Cell lysates were transferred to a 96-well plate, and relative luciferase units were measured for 5 s in a Wallac Trilux 1450 microbeta luminometer.

GAL4 Hybrid Assays—C2C12 and COS-1 cells were passaged into 12-well plates and transfected at 50–80% confluence with 0.33–1 μg of reporter, G5E1b-LUC, and 0.33 μg of GAL-Nur77 chimeric constructs (GAL-Nur77FL, AB, DE, amino acids 1–110, 110–200, 200–269, 1–160, 55–160, and the GAL chimeric constructs containing mutations in the AB region), or the GAL4 DBD alone in the presence and absence of vectors pSG5-SRC-2 (0.33–0.66 μg) and/or pSG5-RXRγ (0.66 μg) expression plasmid/well, using a DOTAP/DOSPER liposome mixture in HEBS. Transfections were performed in DMEM containing 10% FCS or 5% CST (DMEM containing charcoal-stripped FCS was used for transfections performed with GAL constructs cotransfected with pSG5-RXRγ in the presence and absence of 1 μM 9-*cis*-RA). After 16–24 h the medium was replaced, and the cells were harvested 24–48 h after transfection for the assay of luciferase activity. Each experiment represented at least two sets of independent triplicates to overcome the variability inherent in transfection experiments (37–42).

Plasmids and Primer Sequences—The expression plasmids GAL0 (43), pSG5 (Stratagene), pSG5-RXRγ, and pSG5-RXRγ-ΔAB (CDE) (42) and the reporter plasmids pNBRE3-tk-LUC (31–33) and G5E1b-LUC (44) have been described elsewhere. Generation of full-length mouse Nur77 was performed by reverse transcription-PCR from muscle RNA with *Pfu* DNA polymerase (Promega), using the manufacturer's buffer. All PCR products were cloned into the *Eco*RI site of pSG5 (Stratagene) and then isolated after *Eco*RI digestion and subsequently cloned into pGAL0 and pGEX4T1 (Amersham Biosciences). The primers (GENEWORKS Australia) used for the synthesis of full-length mouse Nur77 were GMUQ-713 5′-GCG **GAA TTC** ACC ATG CCC TGT ATT CAA GCT CAA and GMUQ-714 3′-GCG **GAA TTC** TCA GAA AGA CAA TGT GTC CAT. We then synthesized additional primers to subclone the DE, AB, ABC, and CDE regions into pSG5 and GAL0. The primers used for the DE were GMUQ-715 5′-GCG **GAA TTC** ACC ATG CGG CGG AAC CGC TGC CAG and GMUQ-714 3′. The primers used to synthesize the AB region were GMUQ-713 5′ and GMUQ-716 3′-GCG **GAA TTC** TCA CTC GCT GCC ACC TGA AGC CCC. The primers used to synthesize the ABC region were GMUQ-713 5′ and GMUQ-717 3′-GCG **GAA TTC** TCA CAG GCG GAG GAT GAA GAG. The primers used to synthesize the CDE region were GMUQ-718 5′-GCG **GAA TTC** ACC ATG TGT GCA GTC TGT GGT GAC and GMUQ-714 3′.

We subsequently constructed various subdomains of the AB region by PCR and cloned these segments into the *Eco*RI/*Sal*I site of GAL4. The Nur77AB-aa1–110 was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-713 5′ and GMUQ-719 3′-GCG **GTC GAC** TCA CAC CTG GAA GTC CTC. The Nur77AB-aa110–200 region was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-720 5′-GCG **GAA TTC** ACC ATG TAC GGC TGC TAC CCG and GMUQ-721 3′-GCG **GTC GAC** TCA GCC AGT GGG AGG. The Nur77AB-aa200–269 region was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-722 5′-GCG **GAA TTC** ACC ATG CCC AGC CCC AGC and GMUQ-723 3′-GCG **GTC GAC** TCA CTC GCT GCC ACC TGA AGC CCC. The Nur77AB-aa1–160 region was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-713 5′ and GMUQ-724 3′-GCG **GTC GAC** TCA AAA TGA GCC GTC CCA.

The Nur77AB-aa55–160 region was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-725 5′-GCG **GAA TTC** ACC ATG TTC ATG GAC GGG and GMUQ-724 3′.

We then synthesized several primers for site-directed mutagenesis and used the Stratagene QuikChange multisite-directed mutagenesis kit as per the manufacturer's instructions to produce GAL4-Nur77AB chimeras that simultaneously carried double, triple, and quadruple amino acid mutations. Nur77AB mutant primers were synthesized and 5′-phosphorylated by GENEWORKS Australia. The primers made were S54A/T55A-GMUQ-726 5′-CTG CCC AGC TTC **GCA GCC** TTC ATG GAC GGG, S54P/T55P-GMUQ-727 5′-CTG CCC AGC TTC **CCA CCC** TTC ATG GAC GGG, S142A/T145A-GMUQ-728 5′-TCA GCC CCC **GCA** CCA TCT **GCA** CCC AAC TTC C, and S142P/T145P-GMUQ-729 5′-TCA GCC CCC **CCC** CCA TCT **CCA** CCC AAC TTC C.

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GST Pulldowns—GST and GST fusion proteins were expressed in *Escherichia coli* (BL21) and purified using glutathione-agarose affinity chromatography as described previously (37–42). The GST fusion proteins were analyzed on 10% SDS-polyacrylamide gels for integrity and to normalize the amount of each protein. The Promega TNT-coupled transcription-translation system was used to produce [^{35}S]methionine-labeled Nur77 and coactivator proteins that were visualized by SDS-PAGE. *In vitro* binding assays were performed with glutathione-agarose beads (Sigma) coated with ~500 ng of GST fusion protein and 2–10 μl of [^{35}S]methionine-labeled protein in 200 μl of binding buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 5 μg of ethidium bromide, and 100 μg of bovine serum albumin. The reaction was allowed to proceed for 1–2 h at 4 °C with rocking. The affinity beads were then collected by centrifugation and washed five times with 1 ml of binding buffer without ethidium bromide and bovine serum albumin. The beads were resuspended in 20 μl of SDS-PAGE sample buffer and boiled for 5 min. The eluted proteins were fractionated by SDS-PAGE, and the gel was treated with Amersham Amplify fluor, dried at 70 °C, and autoradiographed.

Molecular Modeling—A model of the Nur77 LBD was constructed using the program Modeler 6.0 with the RARγ LBD as a template. The resulting model was subject to Ramachandran analysis and further quality checking with the WhatIf suite of programs (www.cmbi.kun.nl/swift/whatcheck/). Hydrophobic surface analysis was conducted in SCULPT 3.0. Initial peptide superimposition was achieved using SPDBV V3.7, and structures were raytraced with the freeware program Persistence of Vision:POV-Ray (www.povray.org) and the Macintosh patch MacmegaPov (users.skynet.be/cgi-bin/formd.cgi). Further analysis of peptide docking using a spherical polar Fourier correlation docking simulation program was performed using the program HEX (47–49).

RESULTS

Nur77 Trans-activates Gene Expression in a Cell-specific Manner—Nur77 is expressed in skeletal muscle tissue and cells (23, 24, 26, 27). We performed reverse transcription-PCR experiments using total RNA extracted from C2C12 skeletal muscle cells to isolate full-length Nur77 cDNA. To verify the integrity of the cloned NR after full-length sequencing we examined the ability of Nur77 to trans-activate a Nur77-dependent reporter gene, NBRE3-tk-LUC (NBRE derived from the monomeric consensus binding site AAAGGTCA). This plasmid contains three copies of a consensus binding site (AAAGGTCA) cloned upstream of the heterologous herpes simplex virus thymidine kinase promoter (31–33) linked to the luciferase gene. We transfected both myogenic and non-muscle cell types to investigate cell-specific aspects of NR4A1 function.

We investigated the ability of full-length Nur77 and Nur77 lacking the AB region (Nur77ΔAB construct, which encodes amino acids 269–601) to trans-activate NBRE3 in muscle and non-muscle cells in the absence of agonists. In control studies, C2C12 myogenic cells and COS-1 cells were transfected with the control reporter plasmid ptk-LUC (lacking an NBRE, in an pGL2basic backbone), and Nur77 expression plasmids did not trans-activate the empty LUC reporter plasmid in either cell type (Fig. 1, *A* and *B*). However, when C2C12 muscle cells and COS-1 cells were cotransfected with the expression vectors Nur77, Nur77ΔAB, and the reporter plasmid NBRE3-tk-LUC, we observed muscle-specific Nur77-dependent trans-activation of gene expression (Fig. 1, *C* and *D*). Moreover, these experiments suggest that the LBD (DE) region of Nur77 is not sufficient to mediate trans-activation of gene expression (Fig. 1*C*). In summary, the data suggest that Nur77 trans-activated gene expression in a cell-specific manner and that efficient trans-activation requires the AB region, which encodes AF-1.

The AB (AF-1) Region of Nur77 Is Necessary for Agonist-independent and Nur77-mediated Trans-activation: AF-1 Functions in a Cell-specific Manner—The previous experiments suggested that the Nur77 LBD, unlike other orphan and classical NRs, could not exclusively facilitate trans-activation and that Nur77 operated in an AF-1-dependent manner. We went on to examine the role of the AB region in Nur77-mediated trans-activation from the NBRE3-tk-LUC plasmid. We investigated and compared the ability of full-length Nur77, Nur77 lacking the AB region (Nur77ΔAB construct containing amino acids 269–601), and Nur77 lacking the DE region (Nur77ΔLBD construct encoding amino acids 1–356) to trans-activate NBRE in muscle and non-muscle cells in the absence of agonists. Cell specificity has been found to play an important role in the activation functions of the AB and DE domains in the estrogen, glucocorticoid, progesterone, and retinoic acid receptors (45, 46). The ability of different AFs to function has been found to (i) vary in relation to the cell line used and (ii) depend on the spatio-temporal expression pattern of the specific receptor, indicating that cell-specific activation mechanisms are involved in the functioning of the different AFs.

Nur77 is expressed in skeletal muscle (23, 24, 26, 27). Hence, we investigated whether the AB region that encodes the AF-1 domain of Nur77 independently activates gene expression in a cell-specific fashion. C2C12 myogenic cells were cotransfected with receptor expression vectors Nur77, Nur77ΔLBD, and Nur77ΔAB, and the reporter plasmid pNBRE-tk-LUC. As expected, Nur77 trans-activated gene expression ~10-fold in C2C12 cells, and Nur77ΔAB did not activate transcription in C2C12 or COS-1 cells (Fig. 1, *E* and *F*, respectively). Surprisingly, the Nur77ΔLBD construct trans-activated gene expression ~10-fold in a cell-specific manner, as efficiently as the native receptor.

These experiments indicated that the AB region, which encoded the AF-1 domain, was necessary for optimal Nur77-dependent trans-activation of NBRE-dependent reporters in C2C12 muscle cells. Furthermore, and more importantly, these experiments clearly demonstrate that the AB region, which encodes the AF-1 of Nur77, functions in a cell-specific manner and operates in an AF-2/LBD-independent manner.

The AB Region of Nur77 Encodes a Potent AF-1 Domain Located between Amino Acid Positions 1 and 160—To identify and further characterize the domains of Nur77 involved in transcriptional activation, we utilized the GAL4 hybrid system, whereby putative activation domains are fused to the DBD of the yeast transcription factor GAL4 (43). If these regions encode modular activation domains, they complement the GAL4 DBD (to produce a functional trans-activator) and induce the transcription of the GAL-responsive reporter construct G5E1b-LUC, containing an E1b TATA box with five 17-mer GAL4 binding sites linked to the luciferase gene reporter. The system utilized an SV40 promoter expression vector pGAL4-DBD (43), which contains a multiple cloning site downstream of the GAL4 DBD. We fused Nur77 and various domains (*e.g.* AB or DE regions) of Nur77 to the GAL4 DBD and examined the ability of these chimeras to regulate the expression of the G5E1b-LUC reporter in C2C12 and COS-1 cells. The GAL4-Nur77 chimera containing the full open reading frame of Nur77 activated transcription ~100-fold above the control, pGAL0 (GAL4 DBD) in a cell-specific manner (Fig. 2, *A* and *B*). The GAL-Nur77AB plasmid, which contains only the AB regions of Nur77, with the DBD and LBD deleted, increased transcription of the reporter construct ~200-fold over the GAL DBD alone, in myogenic cells, and >1,000-fold in non-muscle cells (Fig. 2, *A* and *B*). This indicated that the AB region of Nur77 contained a potent ligand-independent AF-1 domain. Interestingly, in the absence of its native DBD and LBD, and outside the context of the native receptor in the GAL4 chimera, the activity of the AF-1 domain was not restricted to muscle.

The GAL-Nur77DE, which encodes the LBD of Nur77 with the AB and C regions deleted, did not activate transcription (Fig. 2, *A* and *B*), which was consistent with the reporter

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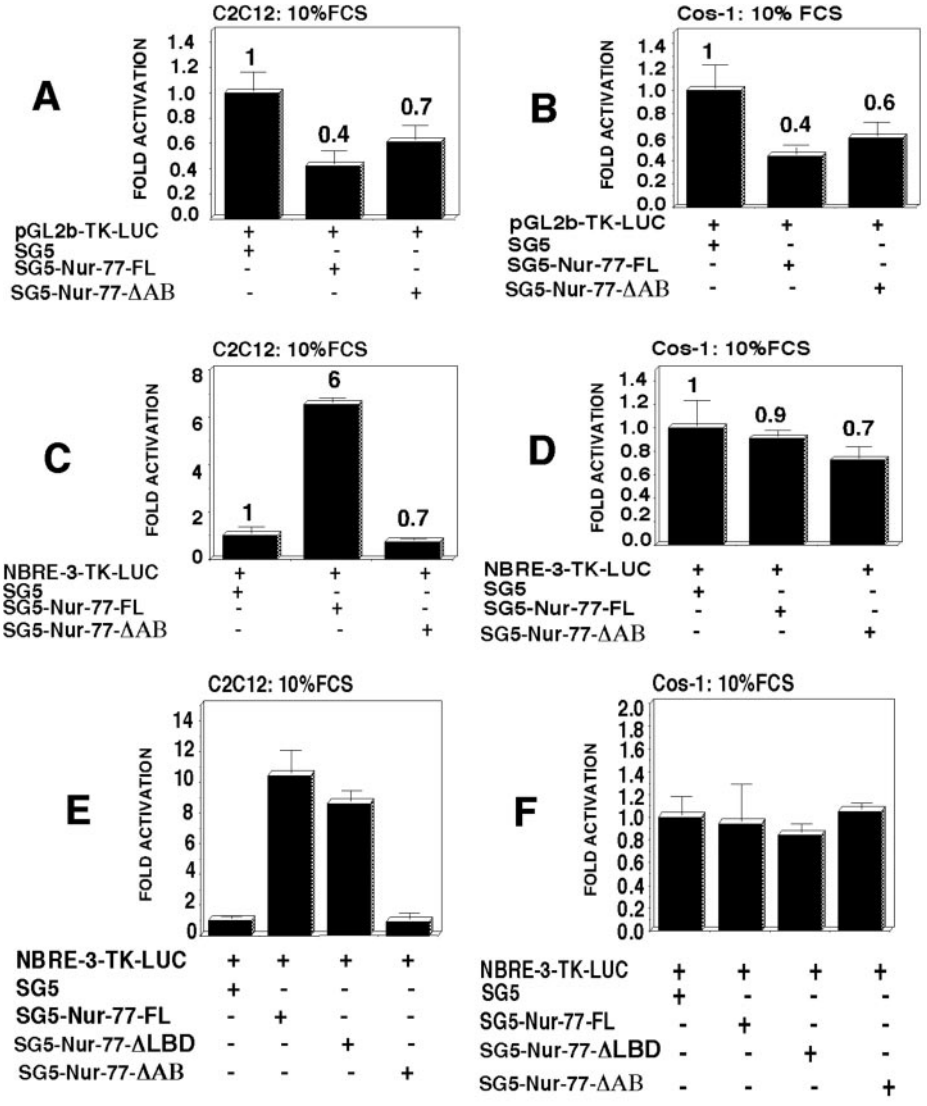

FIG. 1. *A–D*, Nur77 trans-activates gene expression in a cell-specific manner. 0.33 μg of SG5-Nur77FL and SG5-Nur77ΔAB was cotransfected into C2C12 proliferating myoblasts and COS-1 cells together with 1 μg of the reporter genes pGL2b-tk-LUC (*A* and *B*) or NBRE3-tk-LUC (*C* and *D*). *E* and *F*, the AB (AF-1) region of Nur77 is necessary for agonist-independent and Nur77-mediated trans-activation of gene expression. AF-1 functions in a cell-specific manner. 0.33 μg of SG5-Nur77FL, SG5-Nur77ΔLBD, and SG5-Nur77ΔAB was cotransfected into C2C12 proliferating myoblasts (*E*) and COS-1 cells (*F*) together with 1 μg of the reporter gene NBRE3-tk-LUC. -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the pSG5 alone, arbitrarily set at 1. The mean luciferase -fold activation values ± S.D. (*bars*) were derived from a minimum of two or three independent triplicate experiments.

analysis. This suggested that the LBD lacked an intrinsic and classical transcriptional domain.

The AB region is comprised of 269 amino acids, which encodes a potent N-terminal AF-1 domain. To characterize further the AF-1 region of Nur77, we have constructed subregions of the AB domain by PCR and cloned these segments into the GAL4 DBD (Fig. 3*A*). These constructs were transfected into COS-1 cells and assayed in the GAL4 hybrid system (Fig. 3*A*). The constructs GAL-Nur77-aa1–110, GAL-Nur77-aa110–200, and GAL-Nur77-aa200–269 were created and assayed by transfection. These segments of the AB region of Nur77 increased activation 75-, 16-, and 2-fold relative to the control, GAL DBD, alone. This suggested the AF-1 domain was located between amino acid positions 1 and 200 in the AB region. The plasmid, GAL-Nur77-aa200–269, did not trans-activate gene expression in this assay system. These data demonstrated that amino acids 200–269 were not essential for the activity of the AF-1 domain and did not contain an activation function. We subsequently constructed GAL-Nur77-aa1–160 and GAL-Nur77-aa55–160 and assayed these plasmids by transfection analysis. These segments of the AB region of Nur77 increased activation 280- and 44-fold relative to the GAL4 DBD alone. This delimited the AF-1 domain to between amino acid positions 1 and 160 in the AB region and showed that the region downstream of position 160 was not essential for the activity of the AB region.

This delimited domain has two S/T-rich areas between amino acids 50 and 60 and amino acids 140–150 which are highly conserved among the three members of the NR4A subgroup (Nur77, Nurr1, and NOR-1), and cross species (*i.e.* mouse and human). Moreover, phosphorylation of Ser-142 has been implicated in the nucleo-cytoplasmic shuttling of Nur77 (7). Consequently, we investigated whether these regions played a role in AF-1 activity. This was investigated by site-specific mutagenesis of Ser-54, Thr-55, Ser-142, and Thr-145 within the AF-1 domain of Nur77. The AB region carrying various combinations of mutants was cloned into the GAL4 hybrid system. We constructed several mutants that carry multiple mutations, GAL4-AB-S54P/T55P, GAL4-AB-S142A/T145A, GAL4-AB-S54A/T55A/T145A, and GAL4-AB-S54A/T55A/S142A/T145A. We

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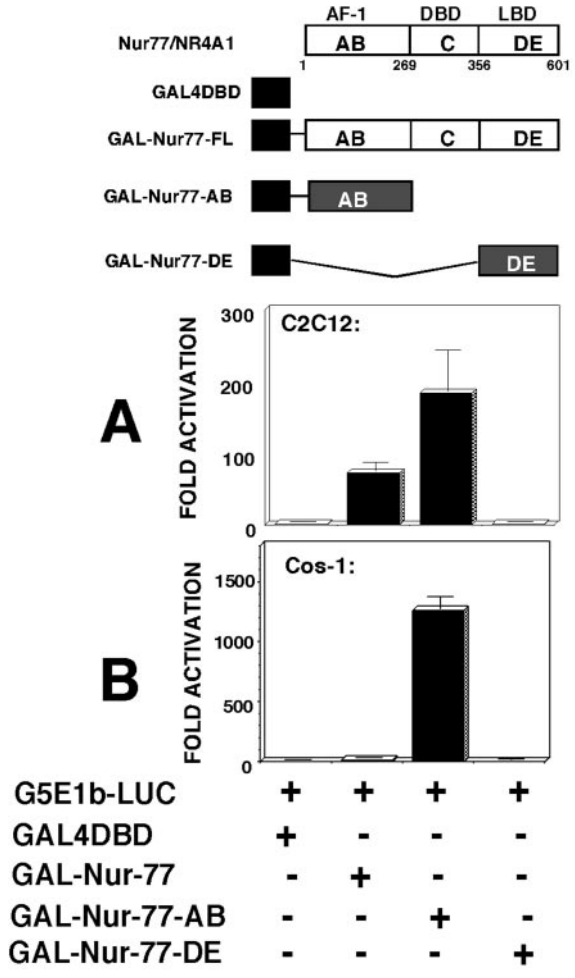

FIG. 2. *A* and *B*, the AB region of Nur77 encodes a potent AF-1 activation domain. 0.33 μg of GAL-Nur77FL, GAL-Nur77AB, and GAL-Nur77DE was cotransfected with 1 μg of the GAL reporter G5E1b-LUC into C2C12 proliferating myoblasts (*A*) and into COS-1 cells (*B*). -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD alone, arbitrarily set at 1. The mean luciferase -fold activation values ± S.D. (*bars*) were derived from a minimum of two or three independent triplicate experiments.

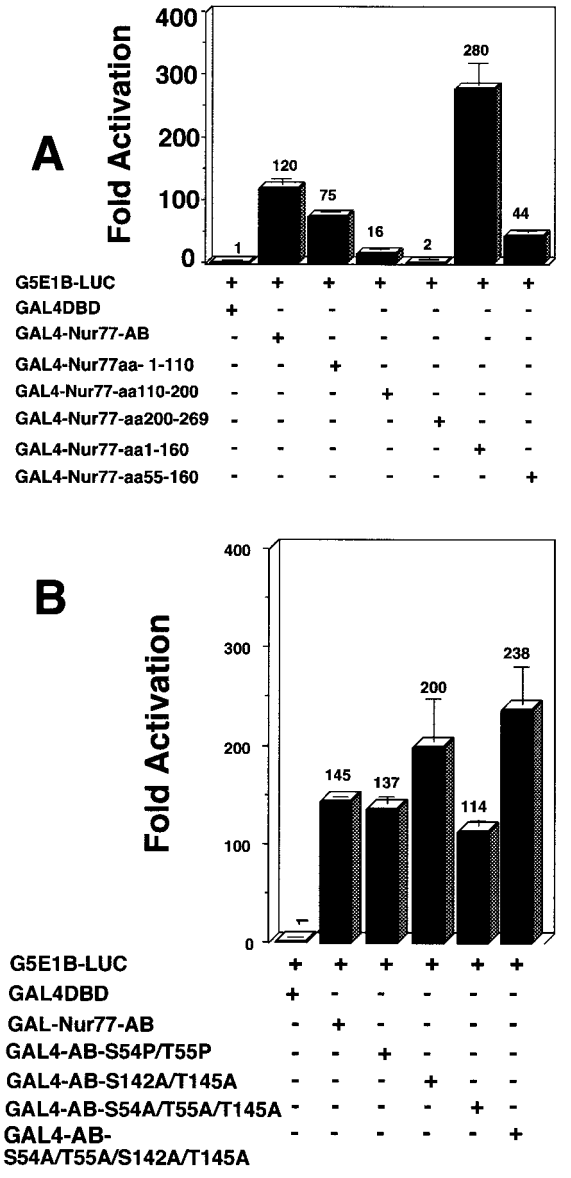

FIG. 3. *A*, the activation domain within the AB region is located between amino acid positions 1 and 160. 0.33 μg of GAL-Nur77AB, GAL-Nur77-aa1–110, GAL-Nur77-aa110–200, GAL-Nur77-aa200–269, GAL-Nur77-aa1–160, and GAL-Nur77-aa55–160 chimeras was cotransfected with 1 μg of the GAL4-dependent reporter G5E1B-LUC into COS-1 cells. *B*, 0.33 μg of GAL-Nur77AB, GAL4-AB-S54P/T55P, GAL4-AB-S142A/T145A, GAL4-AB-S54A/T55A/T145A, and GAL4-AB-S45A/T55A/S142A/T145A (GAL4-Nur77AB chimeras that carried double, triple, and quadruple amino acid mutations, respectively) were cotransfected with 1 μg of the GAL4-dependent reporter G5E1B-LUC into C2C12 cells. -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD alone, arbitrarily set at 1. The mean luciferase -fold activation values ± S.D. (*bars*) were derived from a minimum of two or three independent triplicate experiments.

observed that these mutations had minimal effect on the activity of the AB region in COS-1 cells (data not shown) and C2C12 cells (Fig. 3*B*). However, we observed that simultaneous mutation of Ser-142 and Thr-145 moderately increased the activity of the AF-1.

SRC-2 Stimulates the Activity of the Nur77 AF-1 Domain but Not the Putative LBD—The process of cofactor recruitment and the interaction of coactivators with the NR4A subgroup have remained obscure and have not been described to date. Because Nur77 can activate transcription directly and/or by tethered protein-protein interactions with RXR (31–33), we examined the effect of SRC-2 (GRIP-1) expression on Nur77-mediated trans-activation in the GAL4 hybrid system (Fig. 4). In these assays the activity of Nur77 is independent of its binding to its cognate binding motif, the NBRE. If SRC-2 regulates the transcriptional activity, then the potential of the GAL4-Nur77 fusions to trans-activate gene expression should be increased in this assay (Fig. 4, *A* and *B*).

Cells were cotransfected with GAL-Nur77AB, GAL-Nur77DE, GAL-Nur77-aa110–200, and GAL-Nur77-aa55–160, and the G5E1b-LUC reporter, in the presence and absence of an SRC-2 expression vector. G5E1b-LUC contains five copies of the GAL4 binding site placed upstream of a minimal E1b promoter.

As expected, transfection of GAL-Nur77AB and GAL-Nur77-aa55–160 efficiently induced transcription relative to the GAL4 DBD, and this level of activity was stimulated significantly by 4-fold by the addition of SRC-2. Moreover, GAL-Nur77-aa110–200 was stimulated ~ 10-fold by coexpression of SRC-2. This suggested that SRC-2 modulates the activity of the N-terminal AF-1 domain in Nur77, and this coactivation is dependent on the region between amino acid positions 110 and 160 in the AB region. SRC-2 did not modulate or increase the activity of the C-terminal LBD (Fig. 4*B*).

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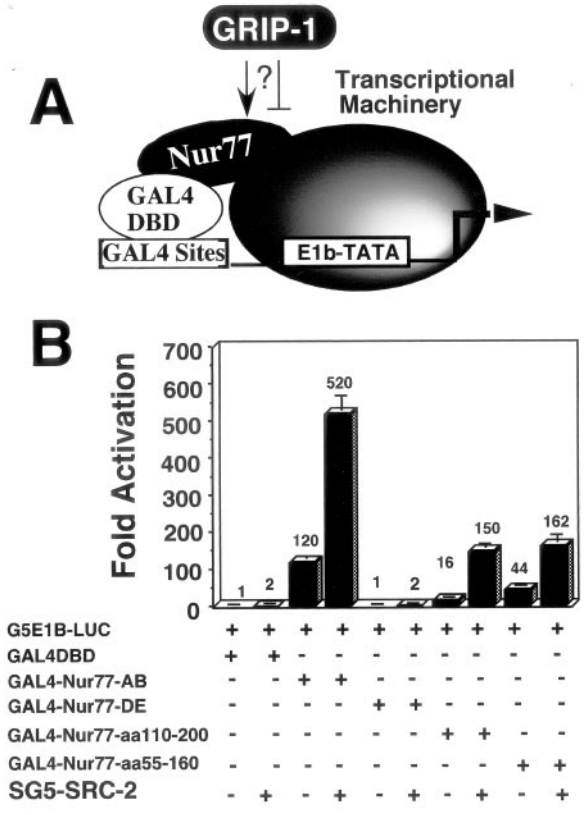

FIG. 4. **SRC-2 potentiates the activity of the Nur77AB region.** *A*, a diagrammatic representation of the GAL4 hybrid assay is shown. This assay was used to determine the effect of SRC-2 (GRIP-1) expression on Nur77-mediated trans-activation of gene expression. *B*, 0.33 μg of GAL-Nur77AB, GAL-Nur77DE, GAL-Nur77-aa110–200, and GAL-Nur77-aa55–160 was cotransfected with 1 μg of the GAL4-dependent reporter G5E1B-LUC into COS-1 cells in the presence and absence of 0.66 μg of the cotransfected expression plasmid encoding SG5-SRC-2. -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD alone, arbitrarily set at 1. The mean luciferase -fold activation values ± S.D. (*bars*) were derived from a minimum of two or three independent triplicate experiments.

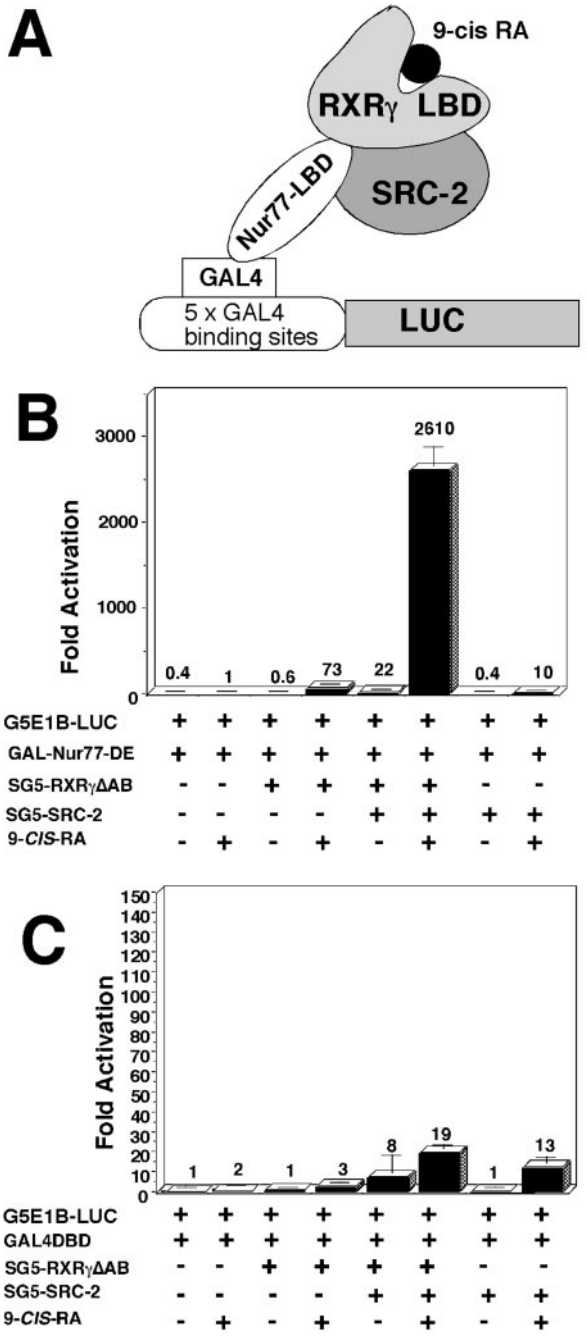

FIG. 5. **SRC-2 stimulates the retinoid-induced RXR-dependent activation of the Nur77 LBD.** *A*, a diagrammatic representation of the GAL4 hybrid assay is shown. This assay was used to determine the effect of SRC-2 (GRIP-1) expression on retinoid-induced RXR-dependent activation of the Nur77 LBD. COS-1 cells were cotransfected with 0.33 μg of GAL-Nur77DE (*B*) or GAL4 DBD (*C*), 0.33 μg of SG5-SRC-2, and 0.66 μg of SG5-RXRγΔAB together with 1 μg of the GAL4-dependent reporter G5E1B-LUC in the presence and absence of 1 μM 9-*cis*-RA. -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD alone, arbitrarily set at 1. The mean luciferase -fold activation values ± S.D. (*bars*) were derived from a minimum of two or three independent triplicate experiments.

SRC-2 Potentiates the Retinoid-induced RXR-dependent Activation of the Nur77 LBD—The NR4A family members can bind as monomers and as homodimers to single/tandem copies of the NBRE, a variant NR half-site, and constitutively regulate transcription (28–30). Moreover, Nur77 and Nurr1 (but not NOR-1) heterodimerize with the C-terminal RXR LBD and mediate efficient trans-activation in response to RXR-specific agonists from a DR5 motif (GGTTCA n_5 AGTTCA) (31–33). The Nur77 LBD lacks an intrinsic activation domain; however, mutations and deletions in the AF-2 domain affect ligand-independent function (32, 33, 36, 50). Moreover, this receptor subgroup has proved refractory to the understanding of coactivator recruitment in the process of trans-activation (50).

Consequently, we examined the ability of SRC-2 to modulate the activity of the GAL4-Nur77 chimeras in the presence and absence of RXRγ (which is preferentially expressed in skeletal and cardiac muscle) (42) and the RXR agonist 9-*cis*-RA (Fig. 5*A*). Cells were cotransfected with GAL-Nur77DE, SRC-2, and RXRγ expression vectors, in the presence and absence of 9-*cis*-RA. As expected, cotransfection of RXRγΔAB in the presence of 9-*cis*-RA increased the activity of the Nur77DE >100-fold relative to the control GAL4 DBD alone (Fig. 5, *B* and *C*). Coexpression of SRC-2 potently and dramatically potentiated the retinoid-induced RXR-dependent activity of the Nur77 LBD from 73-fold (relative to the GAL4 DBD alone) to a dramatic 2,610-fold (relative to the GAL4 DBD), which translates to a ~35-fold increase in the activity of the Nur77 LBD (Fig. 5*B*). In contrast, the activity of the GAL4 DBD alone was increased ~6-fold by retinoids in similar conditions (*i.e.* in the presence of RXR and SRC-2) (Fig. 5*C*). This demonstrated that SRC-2 (GRIP-1) coactivates the efficient Nur77-mediated retinoid-induced RXR-dependent trans-activation.

The Steroid Receptor Coactivators Interact Directly with Nur77: The AF-1 Domain Recruits SRC—The N-terminal AF-1

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region, the C-terminal LBD (and the imbedded AF-2 domain) of the NR4A subgroup, is very unusual and has not been demonstrated to interact directly with coactivators. However, we have demonstrated that SRC-2 modulates the activity of the Nur77 AF-1 domain. Furthermore, activation of gene expression by the classical nuclear hormone receptors is linked to recruitment of SRC-1, -2, and -3. These SRCs recruit p300/CREB-binding protein and PCAF to activate transcription synergistically. SRCs, CREB-binding protein/p300, and PCAF possess intrinsic histone acetyltransferase activity and act in concert to remodel the chromatin. This complex results in the assembly of a higher order structure that includes the DRIP·TRAP·ARC protein complex that regulates localized nucleosome structure (for review, see Ref. 51).

Consequently, we examined the ability of the SRCs (-1, -2, and -3) to interact directly with Nur77. We tested this hypothesis using a biochemical approach, the *in vitro* GST pulldown assay. Glutathione-agarose-immobilized GST-Nur77, GST-Nur77AB, and GST-Nur77DE were tested for direct interaction with *in vitro* ^{35}S-radiolabeled full-length SRC-1, -2, and -3 (Fig. 6*A*). We observed that SRC-1 and -2 interacted efficiently with Nur77. Surprisingly, SRC-1 and -2 interacted directly with the AB region of Nur77, which encoded the potent AF-1 domain. In contrast to other nuclear hormone receptors the DE region that encodes the LBD did not interact with the steroid receptor coactivators (Fig. 6*A*). This is consistent with the NBRE reporter assays and the GAL4 hybrid analysis.

p300 and PCAF Interact Directly with the N-terminal AF-1 Domain: the AB Region Mediates Directs Coactivator Recruitment—To investigate cofactor recruitment further, we examined the ability of p300 and PCAF to interact with Nur77. Glutathione-agarose-immobilized GST-Nur77, GST-Nur77AB, and GST-Nur77DE were tested for direct interaction with *in vitro* ^{35}S-radiolabeled full-length p300 and PCAF. We observed that p300 and PCAF interacted efficiently with Nur77 and Nur77AB (Fig. 6*B*). Surprisingly, p300 interacted very poorly with the C-terminal LBD of Nur77 (Fig. 6*B*). We also investigated the interaction of DRIP-205 with Nur77; however, we did not observe any significant binding (Fig. 6*C*).

As described, SRC, p300, and PCAF interacted independently and directly with GST-Nur77 and GST-Nur77AB. However, we also observed that GST-Nur77 and GST-Nur77AB could simultaneously and efficiently pull down SRC, p300, and PCAF (Fig. 6*D*). In contrast, the DE region that encodes the LBD interacted poorly with this complex. Moreover, we demonstrated that in the presence of SRC-2 and p300, Nur77 and the Nur77 AF-1 interacted significantly with DRIP-205 (Fig. 6*E*).

These studies suggested that the AB region that encodes the AF-1 domain mediates cofactor recruitment in Nur77 and that the LBD interacts poorly with coactivators.

The Nur77 LBD Coactivator Binding Cleft Is Unusually Hydrophilic: Coactivator-derived Peptide Binds Poorly in Docking Simulations—We used molecular modeling as a tool to investigate further the inability of the atypical NR LBD to interact with coactivators. The starting point for homology modeling was the crystal structure of the human RARγ in a complex with the retinoid Cd564 (PDB number 1FCY). Alignments were performed with ClustalV and adjusted manually thereafter. Homology modeling was performed by satisfaction of spatial restraints using the program Modeler6 (47). The resulting model was subjected to Ramachandran analysis and further quality checking with the WhatIf suite of programs. Hydrophobicity analysis was performed using the molecular modeling program SCULPT (48). An L*XX*LL-containing peptide from a previously published thyroid hormone receptor-steroid receptor

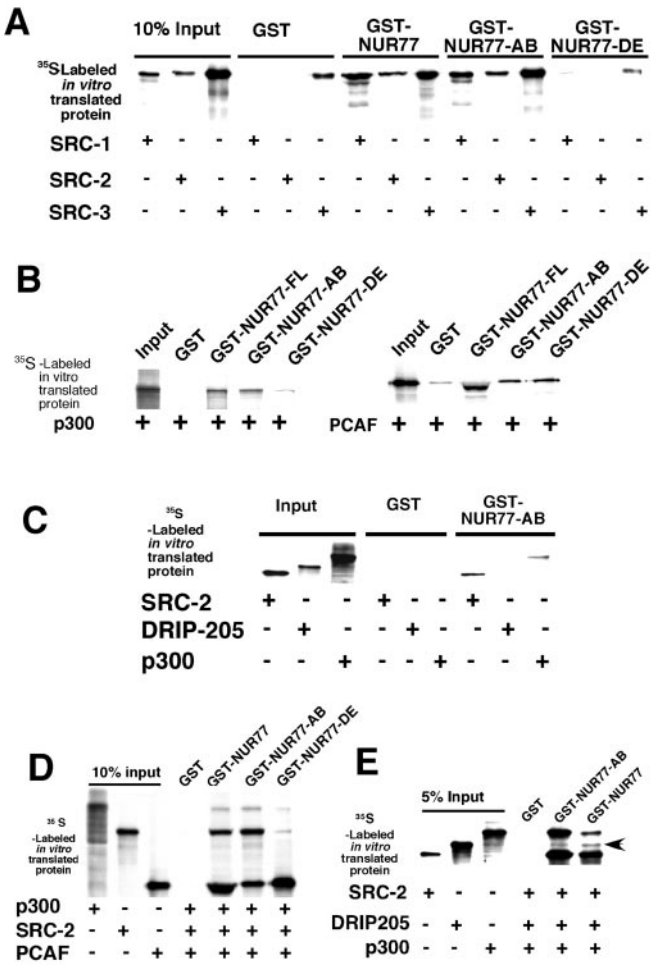

FIG. 6. *A*, SRCs interact directly with the Nur77 AF-1 domain. Glutathione-agarose-immobilized GST, GST-Nur77, GST-Nur77AB, and GST-Nur77DE were incubated independently with *in vitro* ^{35}S-radiolabeled full-length SRC-1, SRC-2, and SRC-3. The *input lane* represents ~10% of the total protein. *B*, p300 and PCAF interact directly with the Nur77 AF-1 domain. GST, GST-Nur77FL, GST-Nur77AB, and GST-Nur77DE were incubated independently with *in vitro* ^{35}S-radiolabeled full-length p300 and PCAF. The *input lane* represents ~10% of the total protein. *C*, GST and GST-Nur77AB were incubated independently with *in vitro* ^{35}S-radiolabeled full-length SRC-2, DRIP-205, and p300. The *input lane* represents ~10% of the total protein. *D*, GST, GST-Nur77, GST-Nur77AB, and GST-Nur77DE were incubated simultaneously with *in vitro* ^{35}S-radiolabeled full-length p300, SRC-2, and PCAF. The *input lane* represents ~10% of the total protein. *E*, GST, GST-Nur77AB, and GST-Nur77 were incubated simultaneously with *in vitro* ^{35}S-radiolabeled full-length SRC-2, DRIP-205, and p300. The *input lane* represents ~5% of the total protein.

coactivator peptide complex was superimposed onto both the RARγ and Nur77 LBDs, enabling us to delineate a hypothetical coactivator binding interface.

Fig. 7 shows ribbon and surface views of the template structure from human RAR (*A*), and the modeled Nur77 (*B*). Examination of the molecular surface in the region of the hypothetical coactivator interfaces shows startling differences in hydrophobicity; human RAR (*C*) shows the characteristic hydrophobic groove (*blue*) found in AF-2 activating receptors, whereas Nur77 (*D*) encoded an unusually hydrophilic surface (*red shade*). Additionally, there are conspicuous changes in local surface topography as shown in close-up views (*E* and *F*). This prompted us to use the spherical polar Fourier correlation docking simulation program HEX (49) to assess the ability of the Nur77 LBD to bind a coactivator-derived peptide (*E* and *F*). When we performed this simulation using the LBD of RARγ we found that 6 of the 10 highest scoring solutions docked to

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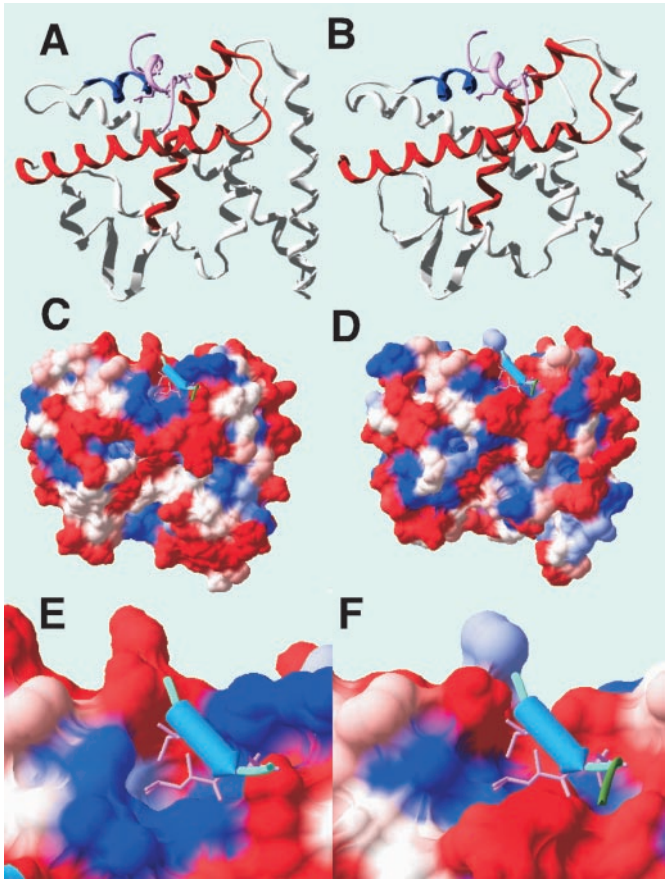

FIG. 7. **Molecular modeling of the Nur77 LBD: comparison of the RARγ LBD with the LBD of Nur77.** RARγ from crystal structure 1FCY (*A*) and the Nur77 (*B*) LBD model and are shown in *ribbon form*. Helices 3–5 (signature motif) are highlighted in *red*, and helix 12 is colored *blue*. An L*XX*LL peptide (colored *pink*) from a previously published thyroid hormone receptor-SRC-2 (GRIP-1) coactivator complex has been superimposed on both structures to delineate a hypothetical coactivator interaction interface. Critical leucines within the coactivator peptide are depicted in *stick form*. The backbone conformations of the two LBDs are very similar. *C* and *D*, hydrophobic analysis of RARγ (*C*) and Nur77 (*D*) molecular surfaces. Surfaces were defined by a probe with a radius of 1.4 Å using SCULPT 3.0. Hydrophobic areas are colored *blue*, and hydrophilic areas are colored *red*. SRC-2 (GRIP-1) coactivator peptide from a previously published structure (55) has been superimposed to delineate an approximate coactivator binding area. Leucines from the coactivator peptide are depicted in *stick form*. A reduction in hydrophobicity is apparent in the Nur77 LBD (*D*) coactivator interface region compared with a similar area of RARγ (*C*). Additionally, the surface topography is different in this region despite the similarity in backbone conformation. *E* and *F*, close-up views of hydrophobic analysis focusing on interaction of leucines from the coactivator peptide with the RARγ (*E*) and Nur77 LBD surfaces (*F*).

within 2 Å root mean square of a coactivator/receptor model based on superposition of the existing thyroid hormone receptor-SRC-2 (GRIP-1) peptide crystal structure. In contrast, the best 100 solutions for Nur77 did not approach to within 5 Å of the equivalent position (data not shown). This is most likely because of the differences in hydrophobicity and topology between the two interfaces. Given the dominance of the SRC-2 (GRIP-1) type L*XX*LL coactivator peptide in receptor interaction, this observation may account for the inability of this NR LBD to mediate cofactor recruitment directly.

The N-terminal AB Region Interacts Directly with the C-terminal LBD: the Interaction Is Potentiated by AF-1-mediated Recruitment of SRC—It has been demonstrated previously that human SRC-2 concomitantly interacts with the estrogen receptor AF-1 and AF-2 to regulate transcription synergistically (52–54). Our studies suggested that the Nur77 AF-1, but not

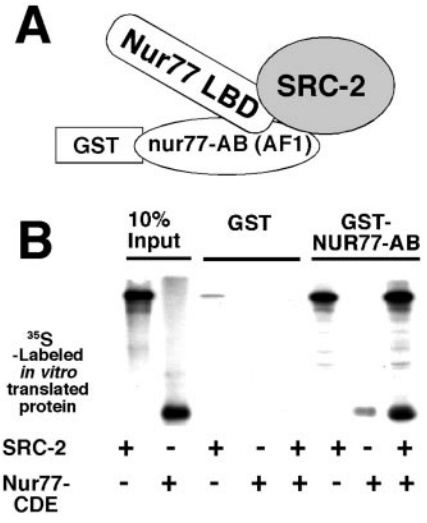

FIG. 8. **AF-1 domain interacts directly with the LBD of Nur77.** This interaction is potentiated by AF-1-mediated recruitment of SRC-2. *A*, diagrammatic representation of the intramolecular interaction between AF-1 and AF-2 of Nur77 which is potentiated by AF-1-mediated recruitment of SRC-2. *B*, GST (*third* and *fourth lanes*) and GST-Nur77AB (*sixth* and *seventh lanes*) were incubated independently with *in vitro* ^{35}S-radiolabeled SRC-2 and Nur77CDE, respectively. GST (*fifth lane*) and GST-Nur77AB (*eighth lane*) were incubated simultaneously with *in vitro* ^{35}S-radiolabeled SRC-2 and Nur77CDE.

the C-terminal LBD, interacts with SRC. We investigated whether the atypical role of the Nur77 LBD in transcriptional activation involved intramolecular interactions with the N-terminal AF-1 and whether this process was modulated by SRC recruitment (Fig. 8*A*).

We tested this hypothesis using a biochemical approach, the *in vitro* GST pulldown assay. Glutathione-agarose-immobilized GST-Nur77AB was tested for direct interaction with *in vitro* ^{35}S-radiolabeled full-length SRC-2 and the Nur77 LBD (CDE) (Fig. 8*A*). As expected from our studies above we observed that SRC-2 interacted efficiently with the Nur77AB. Very interestingly, we observed that the N-terminal AB region that encodes the potent AF-1 domain *interacts directly* with the C-terminal LBD region. Furthermore, the interaction is potentiated significantly by AF-1-mediated recruitment of SRC (Fig. 8*B*). This evidence clearly demonstrates intramolecular interactions between the N-and C-terminal regions of the Nur77 and provides evidence for the involvement of the Nur77 LBD in the absence of an intrinsic activation function. In summary this suggests that SRC-2 potentiates the direct interaction between the N-terminal AF-1 domain and the C-terminal LBD region of Nur77.

The Nur77 AF-1 Domain Synergizes with the RXR LBD in a Retinoid-dependent Manner—In the context of the above data we hypothesized that the Nur77 AF-1 may mediate transcriptional synergy with RXR during retinoid-induced RXR-dependent activation of Nur77 (Fig. 9*A*). Consequently, we examined the ability of RXRγ to potentiate the activity of the GAL4-Nur77 AB plasmid (which encodes AF-1) in the presence and absence of the RXR agonist, 9-*cis*-RA. The experiments were controlled internally with the GAL4 DBD, and GAL4-Nur77DE, which has been well characterized in the literature and in this study with respect to retinoid-induced RXR-specific modulation.

9-*cis*-RA increased the activity of the GAL-Nur77DE chimera in the presence of RXR approximately 728-fold (Fig. 9*B*). In contrast, the activity of the GAL4 DBD alone was increased ~4-fold by 9-*cis*-RA in similar conditions (*i.e.* in the presence of RXRγΔAB) (Fig. 9*C*).

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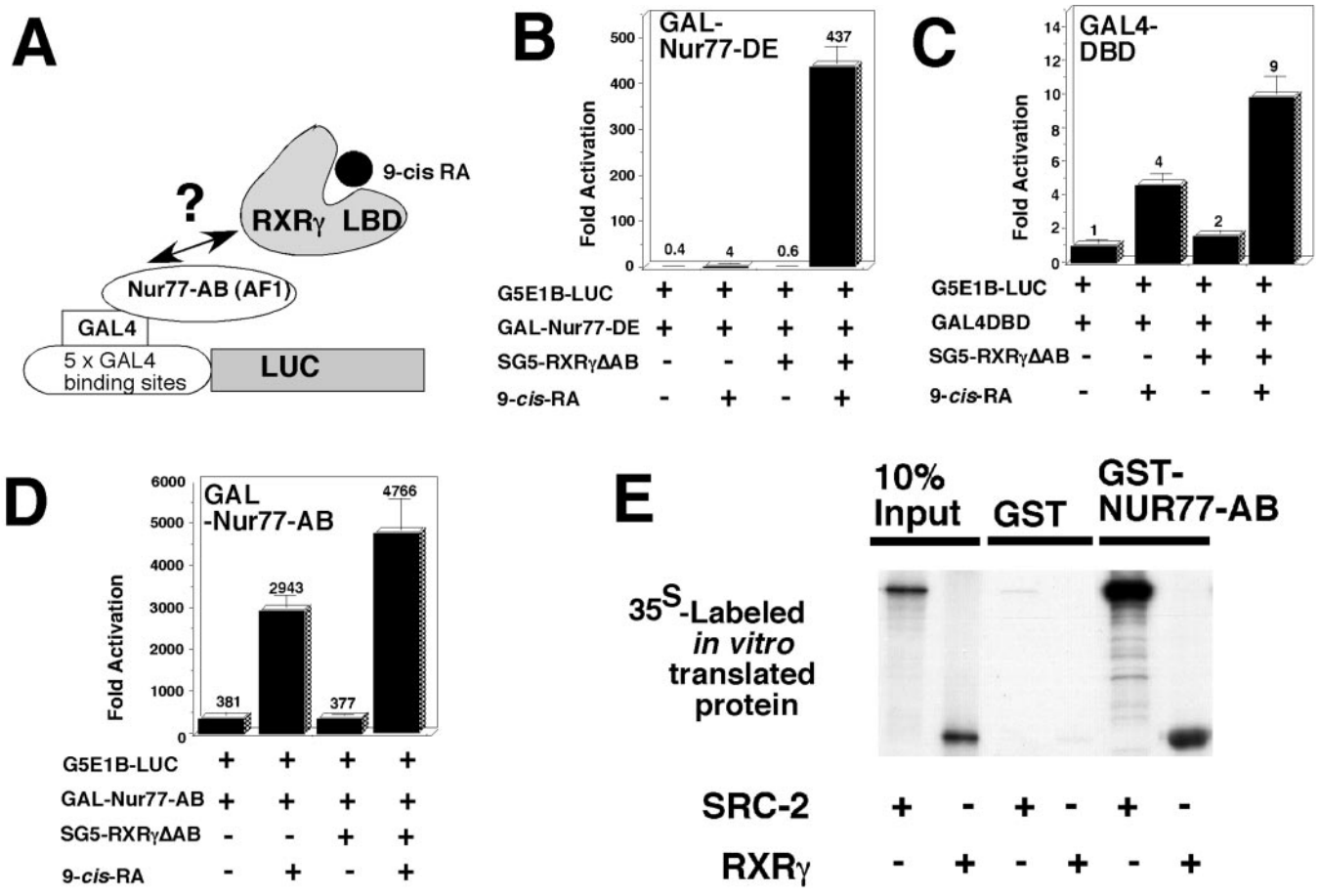

FIG. 9. **AF-1 domain of Nur77 synergizes with RXRγ LBD in a retinoid-dependent manner.** *A*, diagrammatic representation of the GAL4 hybrid assay. This assay was used to determine the effect of retinoid-induced RXR-dependent activation of the Nur77 AB region. *B*, COS-1 cells were cotransfected with 0.33 μg of GAL4-Nur77DE and 0.66 μg of SG5-RXRγΔAB together with 1 μg of the GAL4-dependent reporter G5E1B-LUC in the presence and absence of 1 μM 9-*cis*-RA. *C*, COS-1 cells were cotransfected with 0.33 μg of GAL4 DBD and 0.66 μg of SG5-RXRγΔAB together with 1 μg of the GAL4-dependent reporter G5E1B-LUC in the presence and absence of 1 μM 9-*cis*-RA. *D*, COS-1 cells were cotransfected with 0.33 μg of GAL-Nur77AB and 0.66 μg of SG5-RXRγΔAB together with 1 μg of the GAL4-dependent reporter G5E1B-LUC in the presence and absence 1 μM 9-*cis*-RA. -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the GAL4 DBD alone, arbitrarily set at 1. The mean luciferase -fold activation values ± S.D. (*bars*) were derived from a minimum of two or three independent triplicate experiments. *E*, GST (*third* and *fourth lanes*) and GST-Nur77AB (*fifth* and *sixth lanes*) were incubated independently with *in vitro* ^{35}S-radiolabeled SRC-2 and RXRγ, respectively.

9-*cis*-RA increased the activity of the GAL-Nur77AB chimera in the presence of RXR ~13-fold (Fig. 9*D*). The GAL4 DBD alone was increased ~4-fold by 9-*cis*-RA in similar conditions (*i.e.* in the presence of RXRγΔAB) (Fig. 9*C*). This suggested that the Nur77 AF-1 synergizes very efficiently with RXRγ. We observed stimulation of the Nur77 AF-1 by 9-*cis*-RA in the absence of exogenous RXR, which reflects the well characterized low endogenous expression of RXRs in this cell type (56, 57).

We verified the interaction of the Nur77 AF-1 with RXRγ using a biochemical approach, the *in vitro* GST pulldown assay. Glutathione-agarose-immobilized GST-Nur77AB was tested for direct interaction with *in vitro* ^{35}S-radiolabeled SRC-2 (as a control) and RXRγ (Fig. 9*E*). As expected from our previous experiments, we observed that SRC-2 interacted efficiently with the Nur77AB. Moreover, we observed that the N-terminal AB region that encodes the potent AF-1 domain *interacts directly* withRXRγ (Fig. 9*E*) and is in agreement with the GAL4 analysis.

DISCUSSION

In this investigation we have provided compelling evidence that Nur77-mediated trans-activation operated in an AF-1-dependent manner. The AB region encodes an uncommonly potent N-terminal AF-1 domain delimited to between amino acids 50 and 160, which is essential for the ligand-independent activation of gene expression. Interestingly, the N-terminal AB region (not the LBD) facilitates coactivator recruitment; interacts directly with SRC, p300, PCAF, and DRIP-205; and synergizes with RXRγ in a retinoid-dependent manner. Moreover, we demonstrate that SRC-2 (GRIP-1) (i) modulates the agonist-independent activity of the N-terminal AF-1 domain; (ii) potentiates the retinoid-induced RXRγ-dependent activation of the Nur77 LBD; and (iii) facilitates the physical association of the N-terminal AF-1 with the C-terminal LBD of Nur77. The results imply that the AF-1 domain plays a major role in Nur77-mediated transcriptional activation, cofactor recruitment, and intra- and intermolecular interactions.

Activation by nuclear hormone receptors is mediated by two distinct regions localized within the N- and C-terminal AF-1 and AF-2 regions, respectively. The AF-1 domain is structurally divergent, the AF-2 region is evolutionary conserved, suggesting a general mechanism for transcriptional regulation (1). Accordingly, the majority of receptors regulate gene expression and recruit coactivator complexes in a C-terminal AF-2-dependent manner (51). However, the rat Nur77 LBD (*i.e.* NGFI-B) does not encode an activation domain *per se*. Moreover, the N-terminal of NGFI-B encodes a domain important for transcriptional activity, and this receptor subgroup has

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proved refractory to the elucidation of coactivator recruitment (36, 50).

Specifically, in this study we demonstrate that Nur77 (mouse NR4A1) trans-activates gene expression in a cell-specific manner and in an AF-1-dependent manner. This is consistent with the cell-specific gene expression of NR4A2/Nurr1, and the spatio-temporal specific patterns of expression associated with this gene family (33, 50). This suggests that Nur77 activity is dependent on a cell-specific signal transduction pathway and/or combination of cofactors.

The Nur77 N-terminal AB region encodes an unusually potent AF-1 domain located between amino acids 1 and 160 and is modulated by SRC-2 (GRIP-1). Coactivation by SRC-2 is dependent on the region between amino acids 110 and 160.

The N-terminal AF-1 region, the C-terminal LBD (and the imbedded AF-2 domain) of the NR4A subgroup, as discussed has not been demonstrated to interact directly with coactivators. However, we observed that SRC-2 modulates the activity of the Nur77 AF-1 domain. Because the activation of gene expression by the classical nuclear hormone receptors is linked to the binding of SRCs and the subsequent recruitment of p300/CREB-binding protein and PCAF (51), we investigated coactivator interactions with the GST pulldown assay. We observed that the SRCs, p300 and PCAF interacted directly with the AB region of Nur77 which encoded the potent AF-1 domain. In contrast to other NRs, the DE region that encodes the LBD did not interact with the coactivators, SRC-1, -2, and p300. This is consistent with the NBRE reporter assays and the GAL4 hybrid analysis, which suggested the Nur77 LBD did not contain an intrinsic activation domain. This demonstrated that the N-terminal AB region mediates coactivator recruitment in Nur77 and explains the atypical nature of NR4A-mediated trans-activation. Furthermore, in a cofactor-dependent manner, the AB region of Nur77 and not the LBD interacted with DRIP-205, which is involved in the regulation of nucleosome structure.

Interestingly, we show that the AF-1-mediated recruitment of SRC-2 promotes intramolecular interactions with the LBD which may function to stabilize the receptor during agonist-independent transcription.

Serine/threonine-rich domains in the N terminus have been implicated in the regulation of NGFI-B-dependent transcription (7, 35, 36). Furthermore, the N terminus and phosphorylation of amino acid residues in the AB region are required for the growth factor-dependent nucleo-cytoplasmic shuttling and regulation by extracellular signal-regulated kinase-2, Trk, Ras, and mitogen-activated protein kinase (7, 35, 36). We mutated the serine and threonine residues at amino acid positions 54, 55, 142, and 145, which had previously been implicated as key kinase targets during growth factor-dependent modulation (7, 35, 36). However, these residues were not involved in the transcriptional modulation of this NR in our cell culture systems, and the literature suggests that phosphorylation of these residues mediates nucleo-cytoplasmic transport of this orphan receptor (see below). We did consistently observe a modest increase in the activity of the AF-1 domain when Ser-142 and Thr-145 were mutated simultaneously.

NGFI-B (rat Nur77) and Nurr1 (but not NOR-1) heterodimerize with the C-terminal RXR LBD and mediate efficient trans-activation in response to RXR-specific agonists (31–33). Our study demonstrated that SRC-2 (GRIP-1) potently coactivates the retinoid-induced RXRγ-dependent trans-activation of Nur77-mediated transcription. Moreover, we provided evidence for transcriptional synergy between the Nur77 AF-1 and the RXRγ LBD in a 9-*cis*-RA-dependent manner. These intermolecular interactions mediated by the Nur77 AF-1 with the LBD of RXRγ are consistent with the observations that growth factor-dependent phosphorylation of the N-terminal AB region of Nur77 regulates nucleo-cytoplasmic translocation of this NR and the modulation of retinoid signaling (7).

Our molecular modeling analysis of the NR4A1 (Nur77) C-terminal DE region structurally substantiates the inability of this orphan LBD to interact with coactivators (*e.g.* SRC-2 (GRIP-1)). Examination of the molecular surface in this regions shows an unusually hydrophilic surface that is, in contrast to the archetypal and characteristic hydrophobic groove found in AF-2-activating receptors (*e.g.* RAR and thyroid hormone receptor). Computer modeling strongly suggests that the Nur77 LBD is unable to bind a coactivator-derived peptide because of the differences in hydrophobicity and topology between the Nur77 and classical NRs.

These observations are also consistent with the observation that the AF-2 core regions in the ligand-dependent receptors (*e.g.* RAR and RXR) contain a very highly conserved glutamic acid. This glutamic acid is important for cofactor recruitment and transcriptional activation (50). In contrast the NR4A subgroup (Nur77, Nurr1, and NOR-1) have a conserved lysine.

In summary, we have characterized the functional role of AF-1 in mouse NR4A1 (Nur77)-mediated trans-activation; further elucidated the molecular basis of retinoid-induced RXR-dependent trans-activation by Nur77; and provided a transcriptional, biochemical, and structural analysis of coactivator function and recruitment.

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